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Use of *Wisteria floribunda* agglutinin affinity chromatography in the structural analysis of the bovine lactoferrin N-linked glycosylation ☆

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ABSTRACT

Background: Over the years, the N-glycosylation of both human and bovine lactoferrin (LF) has been studied extensively, however not all aspects have been studied in as much detail. Typically, the bovine LF complex-type N-glycans include certain epitopes, not found in human LF N-glycans, i.e. Gal(α1-3)Gal(β1-4)GlcNAc (αGal), GalNAc(β1-4)GlcNAc (LacdiNAc), and *N*-glycolylneuraminic acid (Neu5Gc). The combined presence of complex-type N-glycans, with αGal, LacdiNAc, LacNAc [Gal(β1-4)GlcNAc], Neu5Ac (*N*-acetylneuraminic acid), and Neu5Gc epitopes, and oligomannose-type N-glycans complicates the high-throughput analysis of such N-glycoprofiles highly.
Methods: For the structural analysis of enzymatically released N-glycan pools, containing both LacNAc and LacdiNAc epitopes, a prefractionation protocol based on *Wisteria floribunda* agglutinin affinity chromatography was developed. The sub pools were analysed by MALDI-TOF-MS and HPLC-FD profiling, including sequential exoglycosidase treatments.
Results: This protocol separates the N-glycan pool into three sub pools, with (1) free of LacdiNAc epitopes, (2) containing LacdiNAc epitopes, partially shielded by sialic acid, and (3) containing LacdiNAc epitopes, without shielding by sialic acid. Structural analysis by MALDI-TOF-MS and HPLC-FD showed a complex pattern of oligomannose-, hybrid-, and complex-type di-antennary structures, both with, and without LacdiNAc, αGal and sialic acid.
Conclusions: Applying the approach to bovine LF has led to a more detailed N-glycome pattern, including LacdiNAc, αGal, and Neu5Gc epitopes, than was shown in previous studies.
General significance: Bovine milk proteins contain glycosylation patterns that are absent in human milk proteins; particularly, the LacdiNAc epitope is abundant. Analysis of bovine milk serum proteins is therefore excessively complicated. The presented sub fractionation protocol allows a thorough analysis of the full scope of bovine milk protein glycosylation. This article is part of a Special Issue entitled Glycoproteomics.

1. Introduction

Lactoferrin (LF) is an 80 kDa, single subunit N-glycoprotein with multiple functions and involved in many processes, such as activation of the immune system [1–4] and inflammatory regulation [5–7], but also iron absorption in the intestinal tract [8], or in prevention of reactive oxygen species formation [9], and growth promotion in certain tissues [10,11]. Furthermore, LF has been shown to have inhibitory effects on tumourigenesis [12,13] and anti-metastatic activity [14–16]. LFs contain between 1 and 5 potential glycosylation sites, depending on the species [17–19]. In human milk, LF is a highly abundant protein, with 5.3 mg/ml in colostrum and around 1 mg/ml in later stages of the lactation [20]. Bovine milk contains a significantly lower amount of LF, with 1.5 mg/ml in colostrum and 20–200 μg/ml in mature milk [21]. There is a sequence homology of 69% between human and bovine LF, and although their 3D structures can be largely superimposed [22], there are some structural differences [23,24]. Human LF consists of 691 amino acids and contains 3 potential glycosylation sites at Asn138, Asn479, and Asn624; Asn138 and Asn479 are occupied with complex-type N-glycans, whereas Asn624 is usually unoccupied [25,26]. Bovine LF consists of 689 amino acids and has 5 potential glycosylation sites at Asn233, Asn288, Asn368, Asn476, and Asn545; Asn233, Asn368, Asn467, and Asn545 are always occupied, and Asn288 is occupied for about 30% [18,19,27].

Abbreviations: 2AB, 2-aminobenzamide; BSA, bovine serum albumin; DMB, 1,2-diamino-4,5-methylenedioxybenzene; FD, fluorescence detection; GRAS, generally recognised as safe; GU, glucose units; HPAEC, high-pH anion-exchange chromatography; HPLC, high-performance liquid chromatography; LF, lactoferrin; MALDI-TOF-MS, matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry; NP, normal phase; PAD, pulsed amperometric detection; PNGaseF, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; SDS, sodium dodecyl sulfonate; WFA, *Wisteria floribunda* agglutinin; WAX, weak-anion-exchange

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For food-related applications, bovine LF, isolated from milk or whey and having the GRAS (Generally Recognised As Safe) status, has widely found use as a humanising protein component for infant formulae. Differences in the glycosylation pattern of human and bovine LF may be a source of concern, considering several structural epitopes present in the bovine LF glycans that do not occur commonly in human glycoproteins, and in particular in the human LF glycans. The Gal(α1-3)Gal epitope (αGal epitope), absent in human glycoproteins, e.g. human LF, but found in bovine glycolipids and glycoproteins, e.g. bovine LF, is an immunogenic structure and has been indicated in allergies [28,29], possibly induced by tick bites [28]. A second epitope that is uncommon in human glycosylation, absent in human LF, but present in bovine LF, is the GalNAc(β1-4)GlcNAc (LacdiNAc) sequence, next to the Gal(β1-4)GlcNAc (LacNAc) epitope (known human glycoprotein examples are urokinase [30], lutropin [31], glycodelin [32], human protein C [33], Tamm-Horsfall glycoprotein [34], and kallidinogenase [35]). Immunogenicity of this epitope has been shown in parasite infections [36], but not in relation to food allergies. Finally, *N*-glycolylneuraminic acid (Neu5Gc), a variant of *N*-acetylneuraminic acid (Neu5Ac), is synthesised by hydroxylation of Neu5Ac at the nucleotide sugar level in all known mammals, except in humans, where a mutation has inactivated the responsible enzyme [37]. However, dietary Neu5Gc can be taken up and used in human glycosylations; it has been found in several human tissues at different levels [38,39].

Glycosylation of bovine lactoferrin was studied previously [40,41], however, no specific focus on LacdiNAc epitopes was made. More sensitive equipment allows for more detailed analysis, as has been done for recombinant human lactoferrin, expressed in the milk of transgenic cows [42]. In this paper we use lectin-affinity chromatography to isolate sub pools of N-glycans, released from bovine LF. By reducing the complexity of glycan mixtures, more detailed analysis of minor compounds is made possible. Using NP-HPLC profiling of 2AB-labelled N-glycans, as well as MALDI-TOF-MS, the lectin-separated sub pools were analysed in native form and after sequential exoglycosidase treatments.

2. Materials and methods

2.1. Materials

Commercial bovine LF was provided by FrieslandCampina DOMO (Amersfoort, The Netherlands). Reference 2AB-labelled N-glycans were obtained from Ludger Ltd (Oxfordshire, UK).

2.2. Monosaccharide analysis

Duplicate samples of ~1 mg bovine LF were subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85 °C) followed by re-N-acetylation and trimethylsilylation. The trimethylsilylated (methyl ester) methyl glycosides were analysed by GLC on an EC-1 column (30 m × 0.32 mm; Alltech, Breda, The Netherlands), using a Chrompack CP9002 gas chromatograph (Chrompack, Middelburg, The Netherlands; temperature program 140–240 °C, 4 °C/min). Monosaccharide derivative identities were confirmed by GLC-MS analysis on a Shimadzu QP2010 Plus system (Shimadzu, 's-Hertogenbosch, The Netherlands), using an EC-1 column (30 m × 0.25 mm, Grace, Breda, The Netherlands) and the same temperature program [43].

Table 1
GU values of monosaccharide constituents determined from glycan standards in this study, compared to GU values of N-glycans from Glycobase 2.0.

	This study	Glycobase 2.0
Core-linked Fuc(α1–6)	0.3–0.5	0.5
Man	0.75–0.95	0.7–0.9
Gal anywhere	0.85–0.95	0.8–0.9
Neu5Ac(α2–3) anywhere	~0.7	~0.7
Neu5Ac(α2–6) anywhere	~1.2	~1.15
GlcNAc (non-bisecting)	~0.7	0.6–0.8

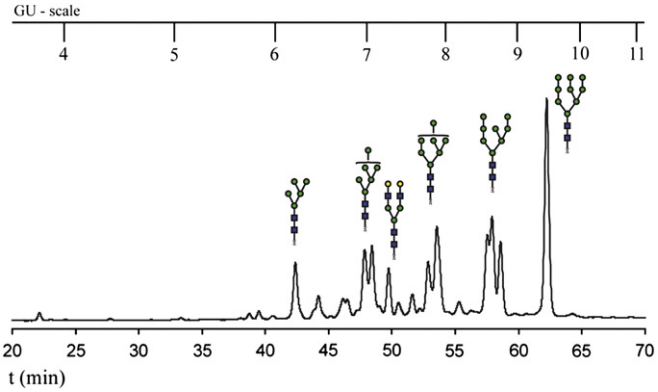


Fig. 1. NP-HPLC profile of 2AB-labelled N-glycans released from bovine LF.

2.3. Sialic acid determination

For HPAEC-PAD analysis, 10 mg bovine LF samples were hydrolysed with 1.0 ml 0.1 M HCl (1 h, 80 °C). Samples were cooled to RT and neutralised with 50 μl 2 M NaOH. Cleaved sialic acid was separated from glycoprotein by 5 kDa centrifugal filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and subsequently analysed on an ICS3000 system (Dionex BV, Amsterdam, The Netherlands), equipped with a CarboPac PA-1 column (4 mm × 250 mm) and an ICS3000 ED pulsed amperometric detector (pulse potentials and durations: E_1 +0.1 V, 410 ms; E_2 −2.0 V, 20 ms; E_3 +0.6 V, 10 ms, E_4 −0.1 V, 60 ms), using a linear gradient of 30–360 mM NaOAc in 0.1 M NaOH. Quantitations of Neu5Ac and Neu5Gc were done by reference to Neu5Ac and Neu5Gc calibration curves, respectively [44].

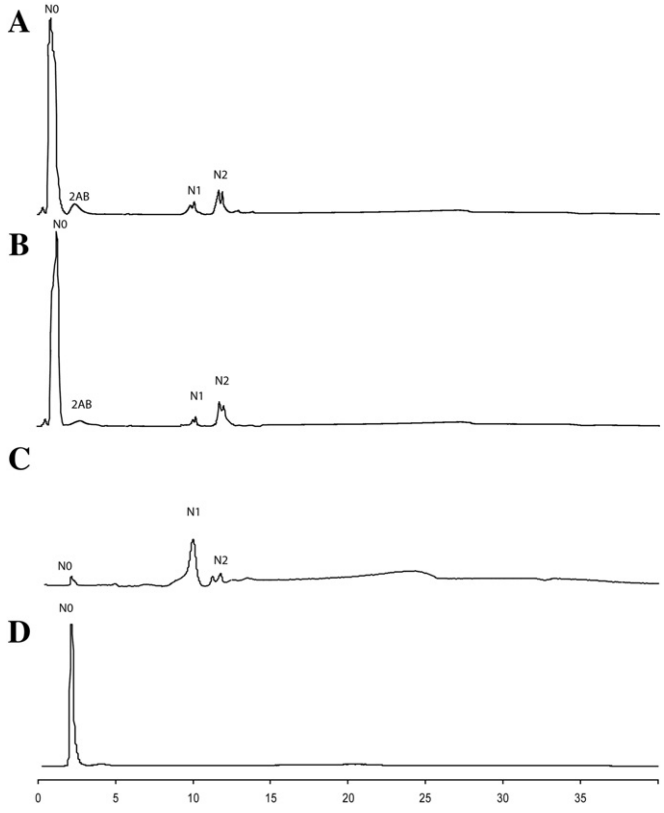


Fig. 2. WAX-HPLC profiles of 2AB-labelled N-glycans of bovine LF. A. total N-glycan pool, B. WFA fraction **I** (unbound), C. WFA fraction **II** (intermediate), D. WFA fraction **III** (bound). N0, neutral glycans; N1, monocharged glycans; N2, dicharged glycans.

For analysis by HPLC-FD, 2 mg bovine LF samples were hydrolysed in 200 μl 2 M propionic acid (3 h, 80 °C). After centrifugation, aliquots of 50 μl supernatant were mixed with 50 μl DMB reagent (7 mM 1,2-diamino-4,5-methylenedioxybenzene, 18 mM sodium hydrosulfite, and 750 mM β-mercaptoethanol in 1.5 M propionic acid) and left to react in the dark for 2.5 h at 50 °C. Analyses were performed on a Waters 2690XE Alliance HPLC system (Waters, Etten-Leur, The Netherlands), equipped with a Waters 474 fluorescence detector (λ_{ex} 373 nm, λ_{em} 448 nm) on a reversed phase Cosmosil 5C18-AR-II column (4.6 mm × 250 mm, Waters, Eschborn, Germany). Elutions were carried

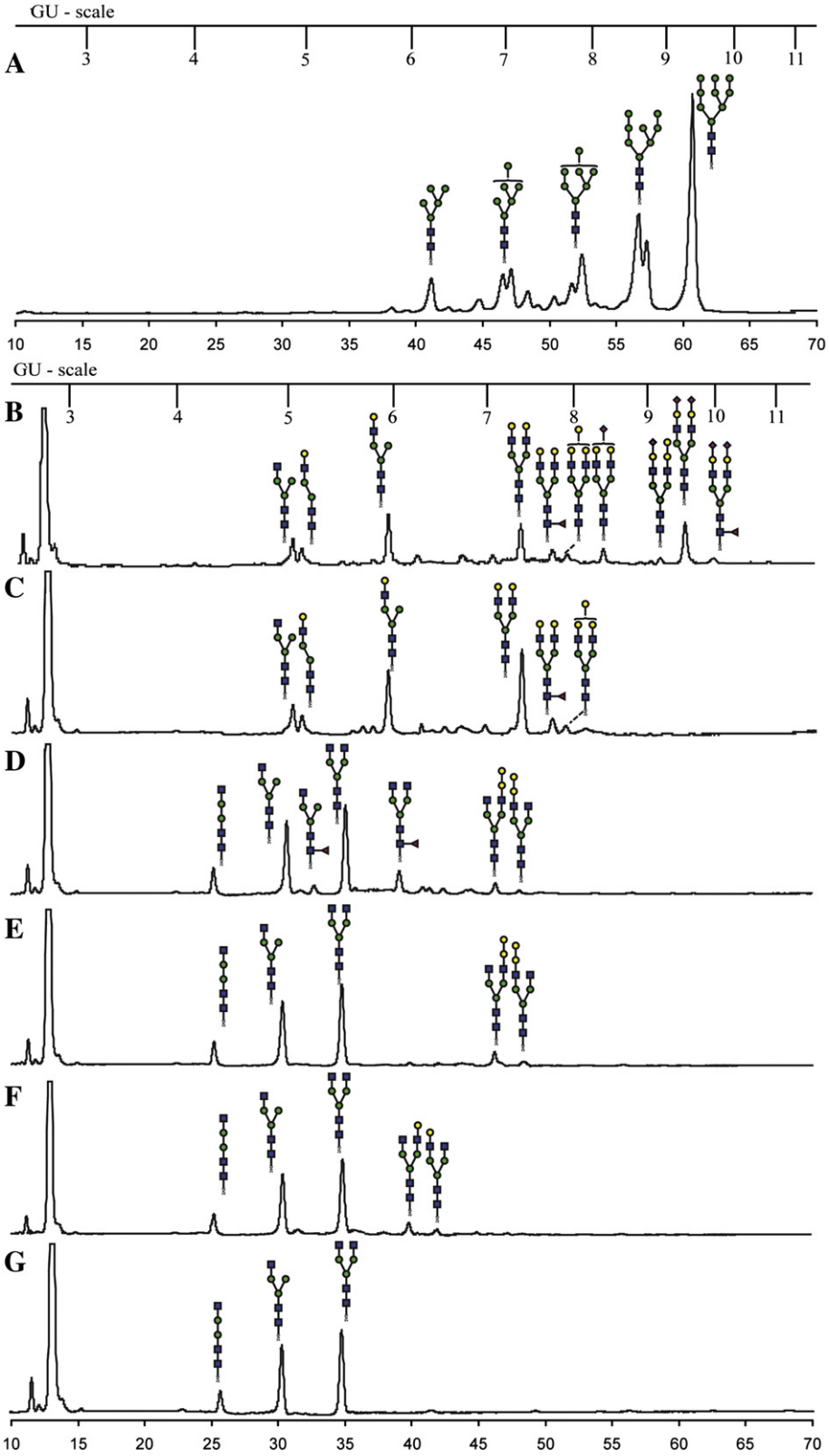


Fig. 3. NP-HPLC profiles of 2AB-labelled N-glycans of WFA fraction **I**; A. without exoglycosidase treatment, and after sequential treatment with B. α-mannosidase, C. α-sialidase, D. β-galactosidase, E. α-fucosidase, F. α-galactosidase, G. β-galactosidase.

out with acetonitrile/methanol/water (9:7:84, v/v/v) as solvent system at a flow rate of 1 ml/min [45].

2.4. N-glycan release

A sample of 50 mg bovine LF was denatured with 100 μl β-mercaptoethanol in 1 ml 1% SDS for 1 h at 85 °C, then 100 μl 1 M iodoacetamide was added, and the mixture was kept for 30 min at 55 °C in darkness. N-glycans were released enzymatically, using peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGaseF) (EC 3.5.1.52) (Roche Applied Science, Mannheim, Germany). First Nonidet P40 substitute (Sigma, St. Louis, MO) was added to a final concentration of 1%. Digestions were performed by adding 15 units of enzyme activity per mg of protein (48 h, 37 °C) in two batches, i.e. 10 U/mg

Table 2
Structures observed in WFA fraction **I** and after sequential exoglycosidase treatments. For each assigned NP-HPLC peak (percentages for each profile A-G are based on HPLC peak integrations), MALDI-TOF-MS confirmation was acquired (*m/z* values are theoretical masses, observed values lie within 1.5 *m/z* range from this value).

Structure	m/z	GU	A[a]	B α	C α 3,6	D β 3,4	E α 2,6	F α 3,4	G β 3,4
	2025.7	9.45	19	0	0	0	0	0	0
	n d [b]	9.12	n d [c]	1	0	0	0	0	0
	1945.8	7.98/8.06	n d	1	1	0	0	0	0
	n d	9.96	n d	1	0	0	0	0	0
	1929.7	7.70	n d	1	1	0	0	0	0
	1863.7	8.80	8	0	0	0	0	0	0
	1863.7	8.71	16	0	0	0	0	0	0
	n d	9.47	n d	7	0	0	0	0	0
	n d	8.34	n d	2	0	0	0	0	0
	1783.7	7.30	7	6	14	0	0	0	0
	1783.7	7.21	0	0	0	1	1	0	0
	1783.7	7.08	0	0	0			0	0
	1701.6	7.91	13	0	0	0	0	0	0
	1701.6	7.85	3	0	0	0	0	0	0
	1621.6	6.42	0	0	0	0	0	1	0
	1621.6	6.32	0	0	0	0	0		0
	1564.6	5.97	0	0	0	1	0	0	0
	1539.5	7.18	5	0	0	0	0	0	0
	1539.5	7.05	4	0	0	0	0	0	0
	1459.5	5.50	0	0	0	15	15	14	15
	1418.5	5.94	n d	7	8	0	0	0	0
	1377.5	6.18	5	0	0	0	0	0	0
	1256.5	5.07	n d	1	1	0	0	0	0
	1256.5	4.95	n d	1	2	9	9	10	9

[a]Exoglycosidase treatments are in sequence: A. Untreated, B. α-mannosidase, C. α-sialidase, D. β-galactosidase, E. α-fucosidase, F. α-galactosidase, G. β-galactosidase.
[b]It should be noted that sialic acids are commonly lost prior to analysis by the MALDI-TOF-MS detector and are therefore not observed.
[c]Prior to treatment with α-mannosidase many of the minor components are not observed, due to overlap with the large oligomannose-derived peaks.

followed by 5 U/mg after 24 h. Released N-glycans were separated from de-N-glycosylated protein on a Carbograph SPE column (300 mg graphitised carbon, Alltech), using 40% aqueous acetonitrile, containing 0.05% TFA as eluent. Then, the obtained N-glycan pool was treated with CalBiosorb beads (CalBiochem, La Jolla, CA), to remove Nonidet P40 and SDS, and Dowex H^+ 50WX8 (Sigma), and passed across a cotton wool plug to remove solids.

2.5. 2AB labelling of N-glycans

The purified and lyophilised N-glycan pool was treated with 0.35 M 2-aminobenzamide (2AB, Sigma), 1 M sodium cyanoborohydride in dimethyl sulfoxide/acetic acid (7:3, v/v) for 2 h at 65 °C. Labelled glycans were purified by paper chromatography, using acetic acid-treated QMA paper (Whatman). Excess 2AB was removed by washing with 8 times 500 μl acetonitrile. Labelled glycans were eluted with 5 times 500 μl Milli-Q water [46,47].

2.6. Lectin affinity chromatography

The lyophilised, 2AB-labelled N-glycan pool was dissolved in 500 μl binding buffer (15 mM PBS, 0.15 M NaCl, pH 7.5) and applied to an agarose-bound *Wisteria floribunda* agglutinin (WFA) column (2 ml bed volume, Vector Laboratories Ltd., Peterborough, UK). After washing with 5 column volumes binding buffer, the lectin column was eluted with 5 column volumes binding buffer/acetic acid (pH 5.5), followed by 5 column volumes 100 mM GalNAc in binding buffer/acetic acid (pH 5.5). The column was reconstituted with 20 mM Tris·HCl, pH 4.5, containing 0.5 M NaCl, and stored in 20% EtOH, 80% 20 mM Tris·HCl, pH 5.8, containing 1 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$.

2.7. Mass spectrometry

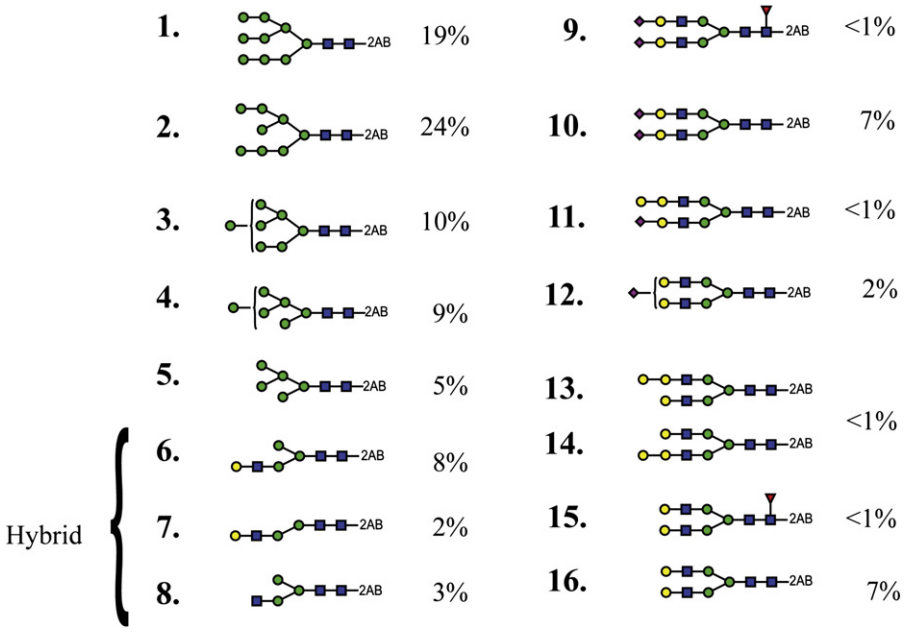
Matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) experiments were performed on an Axima Performance mass spectrometer (Shimadzu Kratos, Manchester, UK) equipped with a nitrogen laser (337 nm, 3 ns pulse width). Positive-ion mode spectra were acquired using the reflectron mode and delayed extraction with software controlled delay time optimised for *m/z* 1500. The accelerating voltage was 20 kV and the acquisition range was 800–4000 Da. 1-μl samples were mixed on the target plate with 1 μl 2,5-dihydroxybenzoic acid (10 mg/ml) as matrix in 40% aqueous acetonitrile. After drying of the droplets, the spots were re-crystallised with ethanol.

2.8. Normal phase-HPLC profiling

2AB-labelled N-glycan samples were profiled on a TSKgel amide-80 column (4.6 mm × 150 mm, 3 μm particle size; Tosoh Bioscience GmbH, Stuttgart, Germany) at 40 °C, using a Waters 2690XE Alliance HPLC system (Waters, Etten-Leur, The Netherlands), equipped with a Waters 474 fluorescence detector (λ_{ex} 330 nm, λ_{em} 420 nm). A 100-min gradient of 50 mM ammonium formate, pH 4.4, in acetonitrile (25–56%) was used at a flow rate of 0.8 ml/min, followed by a 3-min gradient to 100% 50 mM ammonium formate, which was kept for 5 min at 1 ml/min to clean the column, finally followed by a gradient to 25% ammonium formate, which was kept at 0.8 ml/min for 25 min to recondition the column. The column was calibrated with a 2AB-labelled external dextran ladder and reference N-glycans (Ludger Ltd., Oxfordshire, UK), and peak retention times of 2AB-labelled N-glycans were expressed in dextran-ladder-derived glucose units (GU) values. In Table 1 GU values for monosaccharide residues, as determined from our standards, and compared to GU values found in Glycobase 2.0 [48], are presented.

2.9. Exoglycosidase assays

Digestions were performed overnight at 37 °C in 50 mM sodium actetate buffer, pH 5.5. The following enzymes were used: *Arthrobacter ureafaciens* α-sialidase (1 U/100 μl in 10 mM Na_3PO_4, 0.1% Micr-O-protect, pH 7, containing 0.25 mg/ml BSA; Roche, Mannheim, Germany), recombinant *Streptomyces plicatus* β-*N*-acetylhexosaminidase (500 U/100 μl; suspension in 50 mM NaCl, 5 mM Na_2EDTA, pH 7.5; New England BioLabs Inc., Ipswich, MA), jack bean α-mannosidase (65 U/ml in 3.0 M $(NH_4)_2SO_4$, 0.1 mM zinc acetate, pH 7.5, Sigma), bovine testis β-galactosidase (0.2 U/100 μl in 3.2 M $(NH_4)_2SO_4$, pH 5.0; Sigma), green coffee bean α-galactosidase (20 U/100 μl in 3.2 M $(NH_4)_2SO_4$, pH 6.0, containing BSA; Sigma), and bovine kidney α-fucosidase (51 U/100 μl in 3.2 M $(NH_4)_2SO_4$, 10 mM NaH_2PO_4, 10 mM sodium citrate, pH 6.0; Sigma). After digestion the enzymes were removed by 10 kDa cut-off centrifugal filters (Millipore).



Scheme 1. Overview of bovine lactoferrin N-glycan structures found in WFA fraction **I**.

2.10. Weak-anion-exchange chromatography

2AB-labelled N-glycans were analysed by weak-anion-exchange chromatography (WAX) on a DEAE column (Grace Vydac, 4.6 mm × 100 mm, 30 °C), using a Waters 2690XE Alliance HPLC system, equipped with a Waters 474 fluorescence detector (λ_{ex} 330 nm, λ_{em} 420 nm). Separation of charge classes was achieved using a linear gradient of 0–250 mM ammonium formate in 20% acetonitrile. Identification of charged species was achieved by reference to a fetuin N-glycan standard (Ludger Ltd.). Peak areas were used for quantitation.

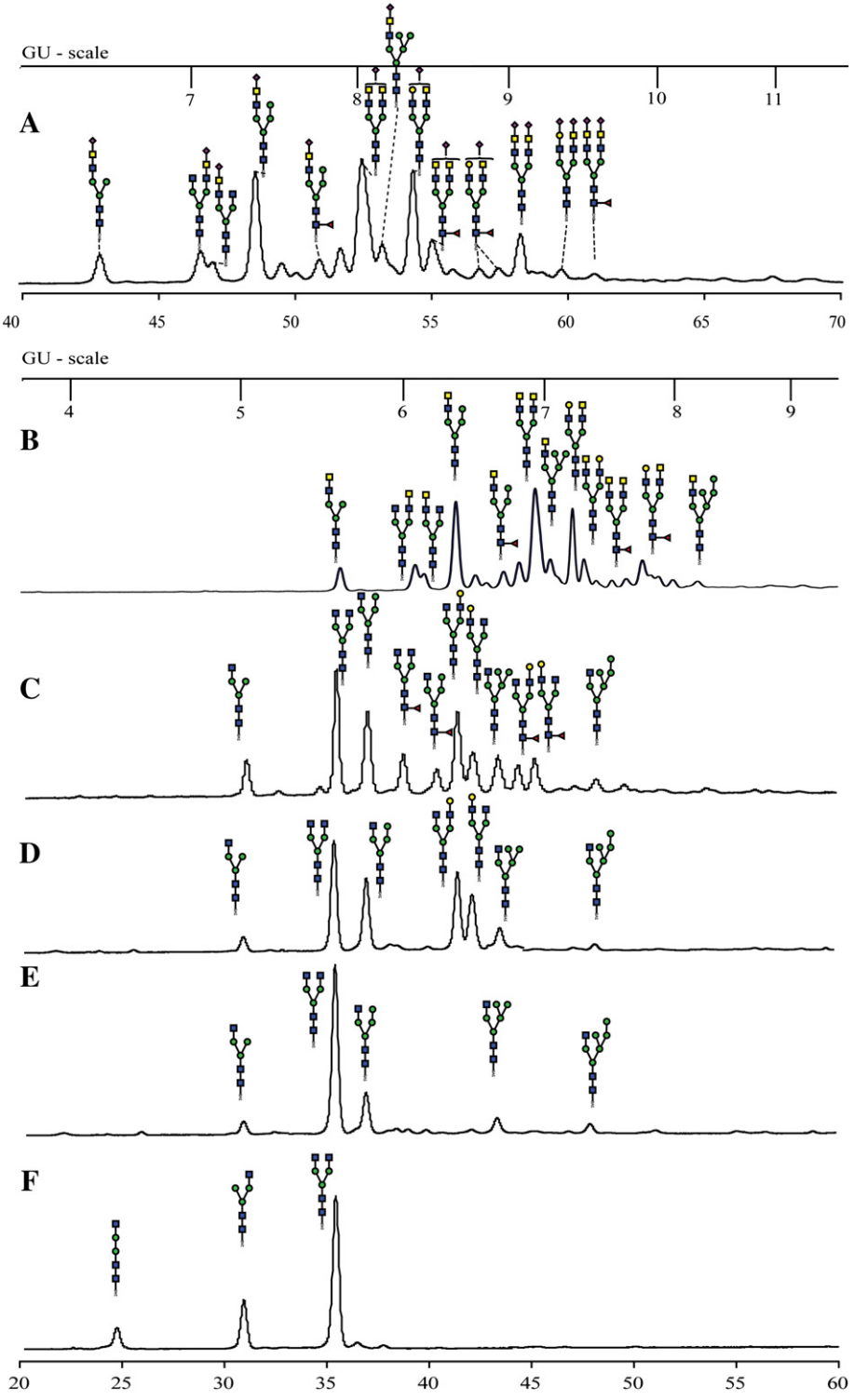
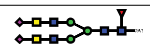
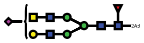
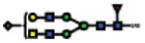
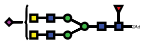


Fig. 4. NP-HPLC profiles of 2AB-labelled N-glycans of WFA fraction **II**; A. without exoglycosidase treatment, and after sequential treatment with B. α-sialidase, C. β-*N*-acetylhexosaminidase, D. α-fucosidase, E. β-galactosidase, F. α-mannosidase.

Table 3
Structures observed in WFA fraction **II** and after sequential exoglycosidase treatments. For each assigned NP-HPLC peak (percentages for each profile A-F are based on peak integrations), MALDI-TOF-MS confirmation was acquired (*m/z* values are theoretical masses, observed values lie within 1.5 *m/z* range from this value).

Structure	m/z	GU	A[a]	B α 3,6	C β 4 β 3,4,6	D α 2,6	E β 3,4	F α
	n d[b]	9.58	1	0	0	0	0	0
	n d	9.34	1	0	0	0	0	0
	n d	9.07	8	0	0	0	0	0
	n d	8.49	6	0	0	0	0	0
	n d	8.41	15	0	0	0	0	0
	n d	8.89	2	0	0	0	0	0
	n d	8.80	2	0	0	0	0	0
	n d	8.54	7	0	0	0	0	0
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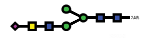
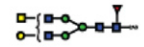
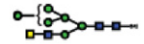
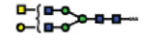
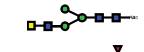
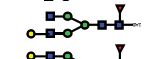
	n d	7.93	4	0	0	0	0	0
	n d	7.38	18	0	0	0	0	0
	n d	7.13	3	0	0	0	0	0
	n d	7.05	5	0	0	0	0	0
	n d	6.68	5	0	0	0	0	0
	1970.7	7.61/7.56	0	5	0	0	0	0
	1945.8	8.16	0	1	0	0	0	0
	2011.8	7.36	0	8	0	0	0	0
	1824.7	7.17/7.10	0	16	0	0	0	0
	1783.7	7.04	0	6	0	0	0	0
	1865.7	6.92	0	27	0	0	0	0
	1621.6	6.83	0	18	0	0	0	0
	1662.6	6.08	0	5	0	0	0	0
	1662.6	6.02	0	3	0	0	0	0
	1459.5	5.56	0	5	0	0	0	0
	1767.7	6.83	0	4	0	0	0	0
	1742.6	7.48	0	0	2	3	3	0
	1767.7	6.89	0	0	5	0	0	0
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	1580.6	6.67	0	0	6	5	6	0
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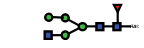
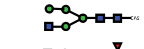
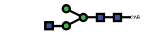
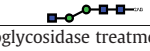
	1564.6	6.21	0	0	3	0	0	0
	1418.5	5.76	0	0	16	18	17	0
	1605.6	5.98	0	0	8	0	0	0
	1459.5	5.55	0	0	31	40	65	66
	1256.5	5.03	0	0	9	9	9	23
	1093.4	4.23	0	0	0	0	0	11

[a]Exoglycosidase treatments are in sequence: A. Untreated, B. α-sialidase, C. β-*N*-acetylhexosaminidase, D. α-fucosidase, E. β-galactosidase, F. α-mannosidase.
[b]It should be noted that sialic acids are commonly lost prior to analysis by the MALDI-TOF-MS detector and are therefore not observed.

3. Results and discussion

3.1. Glycan composition

Monosaccharide analysis [43] of bovine LF showed the presence of Fuc (trace), Man (3.0), Gal (0.4), GalNAc (0.3), GlcNAc (0.7), and Neu5Ac (0.3). The low relative abundances of Gal and GlcNAc in reference to Man, standardised at 3.0 units, are in accordance with large quantities of oligomannose-type structures [40]. Taking into account that O-glycans are absent [40–42], the presence of GalNAc in approximately equal amounts as Gal, indicates a significant occurrence of GalNAc(β1-4)GlcNAc (LacdiNAc) epitopes. It should be noted that the Asn-linked GlcNAc is not cleaved by methanolysis, resulting in an underestimation of GlcNAc. The carbohydrate content of bovine LF was found to be 6.7 +/− 0.2%$_{wt}$. Sialic acid analysis using both HPLC-FD (DMB-labelling; two analyses of duplicate samples) [45] and HPAEC-PAD analysis (triple analysis of duplicate samples) [44] revealed that 8.5 +/− 0.3% of released sialic acid is Neu5Gc. It should be noted that in a pool of bovine milk serum proteins ~1.9% of sialic acid is Neu5Gc [49].

3.2. N-glycan release

In order to develop analytical procedures with one uniform batch of N-glycans, considering the findings of an inter-laboratory study [50,51], a large quantity of bovine LF (100 mg) was chosen for digestion with PNGaseF. The released glycans were isolated by Carbograph SPE chromatography, and screened by MALDI-TOF-MS (data not shown), showing major peaks at *m/z* 1257.4, 1419.5, 1582.1, 1743.6, and 1905.8, corresponding with the sodium adduct masses of $Hex_{5-9}HexNAc_2$. Taking into account the N-glycan biosynthetic pathways in mammals, these peaks indicate a major presence of $Man_5GlcNAc_2$–$Man_9GlcNAc_2$ structures, respectively. Note that for presentation of glycan structures in this report the CFG colour format is used in figures and schemes [52].

3.2.1. NP-HPLC profiling of 2AB-labelled N-glycans

NP-HPLC profiling of the derivatised N-glycan pool (Fig. 1) showed major peaks at GU values corresponding with $Man_5GlcNAc_2$ (GU 6.18), $Man_6GlcNAc_2$ (GU 7.06, 7.18), $Man_7GlcNAc_2$ (GU 7.85, 7.91), $Man_8GlcNAc_2$ (GU 8.71, 8.80), and $Man_9GlcNAc_2$ (GU 9.45), respectively. To eliminate these compounds in the NP-HPLC profile, the N-glycan pool was treated twice with jack bean α-mannosidase, whereby the *N,N′*-diacetylchitobiose core with a single (β1-4)-linked mannose residue attached (GU 2.77) is formed. Integration of the peak at GU 2.77 in relation to the remaining peaks after α-mannosidase treatment, shows a relative abundance of ~65% oligomannose-type structures and ~35% hybrid- and complex-type structures in the N-glycan pool. This observation is in accordance with the relatively high amount of mannose found by monosaccharide analysis, as well as the major MALDI-TOF-MS peaks corresponding with $Man_{5-9}GlcNAc_2$ structures. These findings fit very well with previous reports on bovine LF, showing the occurrence of mainly oligomannose-type glycans [41]. However, so far the presence of relatively large amounts of hybrid-type structures has not been reported.

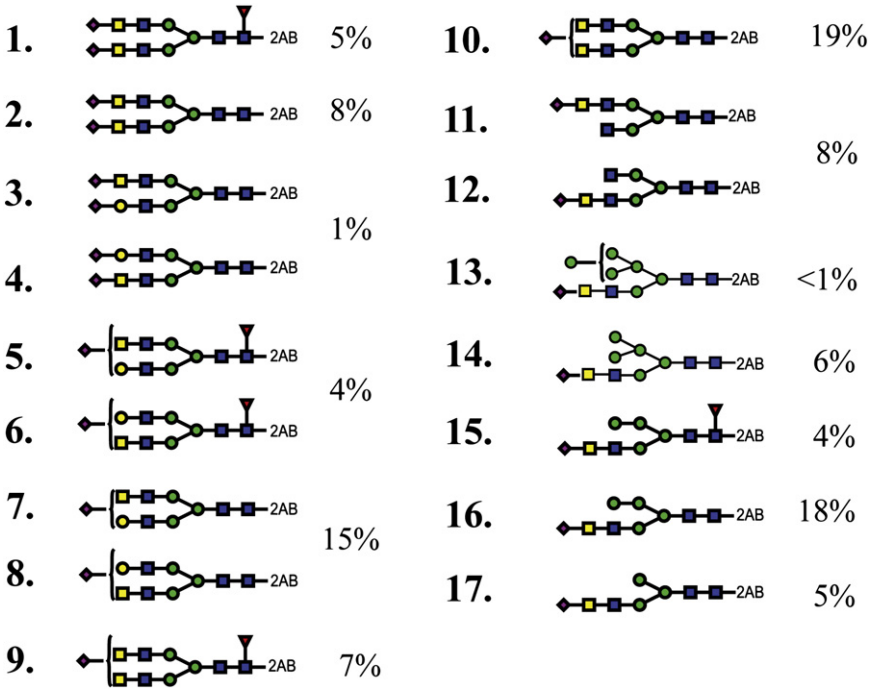
3.2.2. WAX-HPLC profiling of 2AB-labelled N-glycans

Analysis of the charge distribution of the N-glycan pool by weak-anion-exchange chromatography (Fig. 2A) showed a majority of neutral glycans (76%), a significant amount of di-charged structures (15%), with a lower amount of mono-charged structures (9%).

3.2.3. WFA-lectin chromatography combined with NP-HPLC and WAX-HPLC

By using a WFA-agarose column, the N-glycan pool could be separated into three fractions. The non-binding fraction **I** was eluted with binding buffer (PBS/NaCl, pH 7.5), the intermediate fraction **II** with binding buffer, calibrated to pH 5.5 with acetic acid, and the bound fraction **III** with 100 mM *N*-acetylgalactosamine in binding buffer, calibrated to pH 5.5 with acetic acid.

3.2.3.1. WFA fraction **I**. The NP-HPLC profile of WFA fraction **I** showed major peaks corresponding with $Man_{5-9}GlcNAc_2$ (Fig. 3A), together with some minor peaks. WAX-HPLC analysis (Fig. 2B) revealed a large peak for neutral glycans (87%), a peak for mono-charged glycans (3%), and a peak for di-charged glycans (10%). In order to analyse the non-oligomannose-type glycans, all oligomannose-type structures were digested with α-mannosidase (Fig. 3B), resulting in one major



Scheme 2. Overview of bovine lactoferrin N-glycan structures found in WFA fraction **II**.

peak at 2.77 GU (~70%), three peaks corresponding with the remains of hybrid-type structures (~12%), and peaks corresponding with di-antennary complex-type structures (~18%). For a further unravelling of the profile, a series of sequential exoglycosidase treatments was performed. After each exoglycosidase step, a NP-HPLC profile (Fig. 3C to G) and a MALDI-TOF mass spectrum were recorded. In Table 2 a survey of the relative surface area data in the changing NP-HPLC profiles is presented. All included structures, found after each exoglycosidase step, were confirmed by MALDI-TOF-MS. In this way for each HPLC peak in Fig. 3A and B the exoglycosidase steps were followed, yielding the information for elucidating the starting structures.

Using the combined HPLC/exoglycosidase/MS/Glycobase data, the various N-glycan structures in WFA fraction **I** were assigned. A survey of these glycans, together with a rough quantitation within this fraction, based on Fig. 3A and B, is presented in Scheme 1. It has to be noted that many of the structures overlap. Two notable glycans are the minor compounds containing the Gal(α1-3)Gal epitope, one of which, i.e. structure **11** in Scheme 1, was previously found as a major component in a ConA-Sepharose intermediate fraction [41]. It should also be noted that the α-mannosidase treatment at the start of the analysis also degrades all hybrid-type structures (structures **6**–**8**, Scheme 1), thereby limiting the information on hybrid-type structures in this fraction. Considering the abundance of oligomannose-type structures in the N-glycan pool prior to WFA-lectin separation (~65%) and the amount in fraction **I** (~71%), fraction **I** consists of approximately 91% of the LF N-glycans. For the location of Gal(α1-3)- and Neu5Ac(α2-6)- residues,

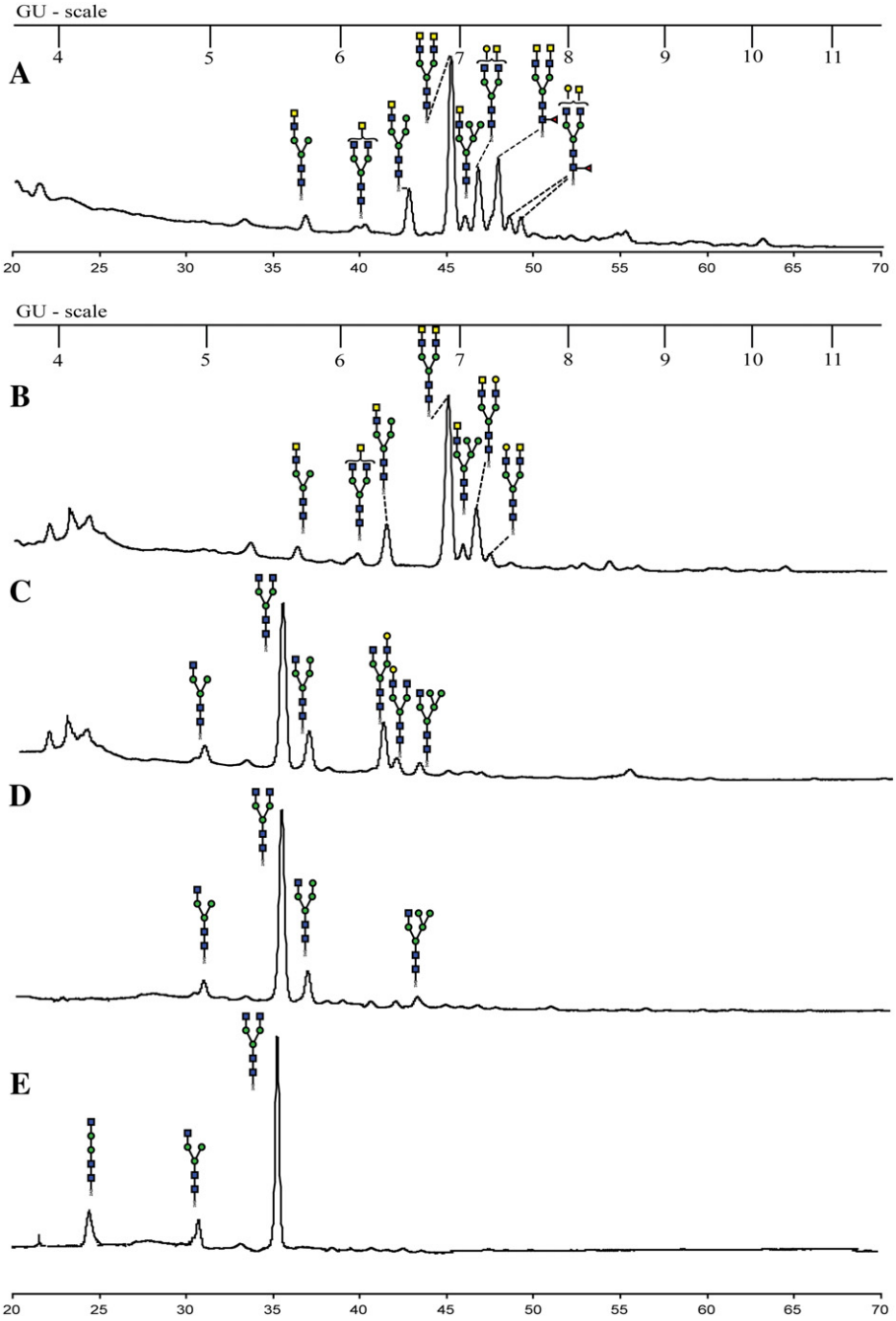


Fig. 5. NP-HPLC profiles of 2AB-labelled N-glycans of WFA fraction **III**; A. without exoglycosidase treatment, and after sequential treatment with B. α-fucosidase, C. β-*N*-acetylhexosaminidase, D. β-galactosidase, E. α-mannosidase.

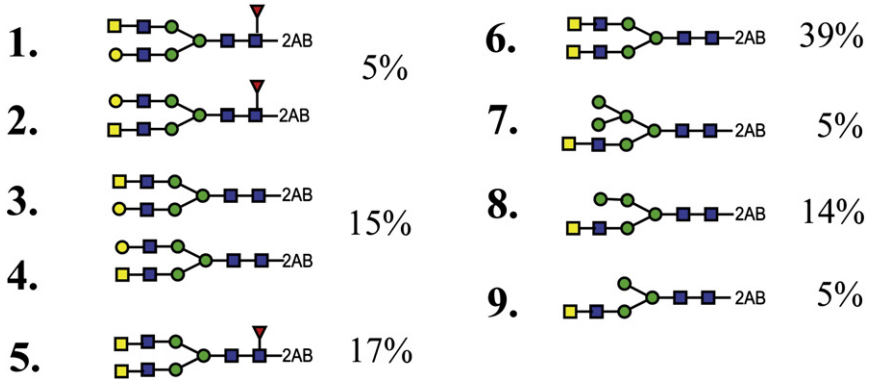
Table 4
Structures observed in WFA fractionS **III** and after sequential exoglycosidase treatments. For each assigned NP-HPLC peak (percentages for each profile A-E are based on peak integrations), MALDI-TOF-MS confirmation was acquired (*m/z* values are theoretical masses, observed values lie within 1.5 *m/z* range from this value).

Structure	m/z	GU	A[a]	B α 2,6	C β 4 β 3,4,6	D β 3,4	E α
	1970.7	7.61/7.56	9	0	0	0	0
	2011.8	7.36	15	0	0	0	0
	1824.7	7.17/7.10	14	22	0	0	0
	1783.7	7.04	5	5	0	0	0
	1865.7	6.92	36	52	0	0	0
	1621.6	6.83	12	13	0	0	0
	1662.6	6.08	2	2	0	0	0
	1662.6	6.02	2	2	0	0	0
	1459.5	5.56	5	4	0	0	0
	1580.6	6.67	0	0	5	5	0
	1621.6	6.49	0	0	6	0	0
	1621.6	6.37	0	0	16	0	0
	1418.5	5.76	0	0	14	15	0
	1459.5	5.55	0	0	53	74	75
	1256.5	5.03	0	0	6	6	9
	1093.4	4.23	0	0	0	0	16

[a]Exoglycosidase treatments are in sequence: A. Untreated, B. α-fucosidase, C. β-*N*-acetylhexosaminidase, D. β-galactosidase, E. α-mannosidase.

biosynthetic pathways and branch specificity of the responsible glycosyltransferases were taken into account [41,53–57].

3.2.3.2. WFA fraction **II**. NP-HPLC analysis of WFA fraction **II** (Fig. 4A) revealed a range of peaks between 6.6 and 10.0 GU. The WAX-HPLC profile (Fig. 2C) showed a trace amount of neutral glycans, a majority of mono-charged glycans (~84%), and a minor amount of di-charged glycans (~16%). In a similar fashion as was done for WFA fraction **I**, all HPLC peaks in Fig. 4A were followed in a series of sequential exoglycosidase treatments (Table 3). After treatment with α-sialidase (Fig. 4B) the three major peaks at 7.38, 8.01, and 8.34 GU shifted to 6.34 (ΔGU 1.04), 6.92 (ΔGU 1.09), and 7.10 (ΔGU



Scheme 3. Overview of bovine lactoferrin N-glycan structures found in WFA fraction **III**.

1.24), respectively, corresponding with the loss of a single (α2-6)-linked Neu5Ac residue. Treatment with *Streptomyces plicatus* β-*N*-acetylhexosaminidase the NP-HPLC profile (Fig. 4C) caused again shifts in all peaks. The peaks at 6.34, 6.92, and 7.10 GU shifted to 5.76 (ΔGU 0.58), 5.55 (ΔGU 1.37), and 6.37 (ΔGU 0.73), corresponding with the loss of one, two and one HexNAc residues, respectively. The enzyme that was selected (*Streptomyces plicatus*) has affinity for GlcNAc(β1-3/4/6)- and GalNAc(β1-4)-linked, leaving the (β1-2)-linked GlcNAc residues of hybrid-type and di-antennary complex-type structures intact. So far, no tri- or tetra-antennary complex-type structures have been reported for LF [40–42], nor have we observed any evidence for such structures in WFA fraction **I**. In view of this absence, the selected enzyme will only cleave GalNAc residues in LacdiNAc epitopes. Combining the NP-HPLC and MALDI-TOF-MS data, collected after each exoglycosidase treatment, and taking into account the Glycobase library data, the structures in WFA fraction **II** were assigned, and are presented in Scheme 2. The quantification of the assigned compounds is based on peaks in NP-HPLC. It should be noted that the intensity of the peaks in NP-HPLC of WFA fraction **II**, taken up in the same amount of Milli-Q water after WFA-separation, is much higher than for fraction **III**, indicating fraction **II** is the major WFA-bound fraction.

3.2.3.3. WFA fraction **III**. The NP-HPLC profile of WFA fraction **III** (Fig. 5A) showed one major peak at 6.95 GU, corresponding with the GU value found in WFA fraction **II** for $[GalNAcGlcNAc]_2$-Man_3-$GlcNAc_2$ (*m/z* 1865.7); its relationship was confirmed by MALDI-TOF-MS (not shown). WAX-HPLC profiling (Fig. 2D) indicated the presence of only a neutral fraction. In the NP-HPLC profile several smaller peaks occurred, corresponding with structures containing a single LacdiNAc epitope, and minor peaks for core fucosylated structures. In a similar fashion as carried out for WFA fractions **I** and **II**, WFA fraction **III** (start profile in Fig. 5A) was subjected to a series of sequential exoglycosidase treatments (Table 4), monitored by NP-HPLC (Fig. 5B–E) and MALDI-TOF-MS. Combining the HPLC/exoglycosidase/MS data and taking into account the biosynthetic pathways, structures were assigned, and are presented in Scheme 3. Again, relative abundances of compounds are based on NP-HPLC profiles.

4. Conclusions

Although the N-glycosylation of bovine LF has been studied extensively before [41], not all aspects were studied in as much detail. First, the report shows a methodology to simplify the analysis of N-glycans in case that besides LacNAc also LacdiNAc units are present in the hybrid- and complex-type oligosaccharides. The inclusion of WFA-lectin affinity chromatography, applied to the released and 2AB-labelled N-glycans, in the usual analysis protocol based on NP-HPLC/WAX-HPLC/exoglycosidases/MS, makes a first unravelling of complex glycan mixtures containing both LacNAc and LacdiNAc elements possible, thereby simplifying the assignment procedures. Well-separated fractions, containing mixtures of glycans with specific structural elements, are obtained, of which the strongly bound fraction contains structures with the LacdiNAc unit, and the weakly bound fraction structures with LacdiNAc units shielded by sialic acid. Secondly, previous studies had not focused on the quantity of Neu5Gc present in this glycoprotein. In the present report it is shown that bovine LF contained 8.5 +/− 0.3% Neu5Gc. This is a relatively high amount, compared to bovine milk and whey protein concentrates (~2.6% and ~1.9%, respectively), however, still lower than reported for ewe and goat milk [49].

In this research a total of 42 N-glycan structures have been assigned for bovine LF; in previous work on bovine LF, only 26 N-glycan structures were reported [41]. For recombinant human LF, expressed in bovine milk, 17 N-glycan structures were published [42]. Structures containing LacdiNAc were observed in all possible forms, with up to two sialylated LacdiNAc units, and even previously unreported LacdiNAc-containing hybrid-type structures. The Gal(α1-3)Gal- epitope was observed in minor amounts in different structures of the unbound bulk fraction; previously, only a single structure as a major component in a ConA-Sepharose intermediate fraction was found [41].

Acknowledgements

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